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Ring-Closure Mechanisms Mediated by Laccase to Synthesize Phenothiazines, Phenoxazines, and Phenazines

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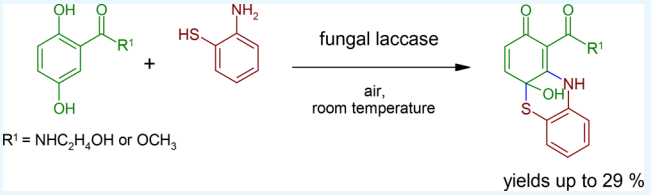
ABSTRACT: The green and environmentally friendly synthesis of highly valuable organic substances is one possibility for the utilization of laccases (EC 1.10.3.2). As reactants for the herein described syntheses, different *o*-substituted arylamines or arylthiols and 2,5-dihydroxybenzoic acid and its derivatives were used. In this way, the formation of phenothiazines, phenoxazines, and phenazines was achieved in aqueous solution mediated by the laccase of *Pycnoporus cinnabarinus* in the presence of oxygen. Two types of phenothiazines (3-hydroxy- and 3-oxo-phenothiazines) formed in one reaction assay were described for the first time. The cyclization reactions yielded C−N, C−S, or C−O bonds. The syntheses were investigated with regard to the substitution pattern of the reaction partners. Differences in C−S and C−N bond formations without cyclization are discussed.



INTRODUCTION

Laccases (EC 1.10.3.2, benzenediol/dioxygen oxidoreductase) are of general interest for biotechnological applications. The enzyme-mediated oxidations demand only atmospheric oxygen and no depletive cofactors such as NADPH. Laccases oxidize substrates by one-electron reactions via four copper atoms, which are situated in the catalytic center.[1−4] The enzyme can oxidize a broad range of substrates such as phenols, thiols, or amines.[5−11] The laccase-mediated oxidations result in the formation of radicals that can undergo three different reaction mechanisms. The first mechanism involves the cleavage, while the second and third mechanisms involve coupling/bond formation. The oxidized laccase substrate can react with molecules of the same kind (homomolecular reaction) or with other radicals or molecules, which can be either laccase substrates or nonlaccase substrates (heteromolecular reaction).[9,12] The homomolecular reaction may result in the formation of C−C,[13−15] C═C,[16,17] C−O,[18,19] N═N,[12] or S−S bonds.[10,11] The heteromolecular reaction allows the combination of compounds via C−C,[20,21] C═C,[17] C−O,[22] C−N,[23−35] C═N,[17,23,25] or C−S.[7,11,16,36] The bond formations result in the synthesis of di-, tri-, and polymers whereby novel ring-closure mechanisms forming cyclic products are also possible but less explored.[7,37−39] These reactions are the basis for the derivatization of biologically active substances such as catechin,[40] mithramycin,[21] pyrimidines,[41] epinephrine (adrenaline),[39] azoles,[33,42] penicillins, and cephalosporins.[27,31]

Other important targets for laccase-mediated reactions are phenothiazines, phenoxazines, and phenazines. Phenothiazines such as chlorpromazine are widely used in medicine as neuroleptics.[43] Phenothiazines, phenoxazines, and phenazines have anticancer[44−46] and antimicrobial[47−49] activities. In addition, phenothiazines have been reported to possess multidrug resistance reverting activity.[50,51] Phenothiazines are also part of dyes such as methylene blue or act as electron donors in photovoltaic cells.[52,53] Phenoxazinones and phenazines can also be used in optoelectronics such as in organic light-emitting diodes.[54,55] Chemical methods to synthesize phenothiazines comprise catalyses with transition metals such as copper iodide, iron salt, or palladium in organic solvents and in part at elevated temperatures above 90 °C.[56−59] Alternatively, KI or Cs_2CO_3 in organic solvents can be used.[60,61] The phenothiazines can be oxidized to the respective phenothiazinones, e.g., by $K_2Cr_2O_7$ or $Na_2Cr_2O_7$ in boiling acetic acid.[62] Other biologically active heterocycles such as benzoxazepines can be synthesized in organic solvents at a temperature of 100 °C.[63] The successful laccase-mediated synthesis of different substance classes (including in part also isomer formation) in comparison with traditional organic reactions catalyzed by oxidants such as silver oxide, sodium iodate, or manganese dioxide was described previously.[24,64−68] This recommends the laccase for various industrial applications.[69,70]

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The utilization of mild and environmentally friendly reaction conditions such as room temperature, atmospheric pressure, and the avoidance of organic solvents makes the laccase-mediated reaction a valuable tool in green chemistry for the synthesis of novel organic compounds as fine chemicals.

The literature of laccase-mediated syntheses is mainly focused on Michael addition reactions. Thus, the reaction of substituted hydroquinones and amines results in mono- or diaminated quinones[24,25,32,67] or quinonimines.[25,71] The formation of cyclic products may be a cascade reaction involving also Michael addition with subsequent 1,2-addition or addition−elimination reaction. After 1,2-addition, also spiro-cyclization or rearomatization is conceivable.[37] Other reactions involve further laccase-mediated oxidations, additional Michael additions, or 1,3-additions.[39,41]

The introduction of newly synthesized substances that were formed during ring-closure mechanisms is a further possibility to broaden the application field for laccase-mediated reactions. Thus, we derivatized substituted *para*-hydroquinones using the laccase-mediated reaction with thiols and amines. The formed products were different homo- and heteromolecular products. The cyclization reactions resulted in the synthesis of phenothiazines, phenoxazines, or phenazines.

Phenoxazines and phenazines are, in contrast to phenothiazines, naturally occurring substances. Thus, fungi of the genus *Pycnoporus* produce orange-red pigments of the phenoxazinone type such as cinnabarin, tramesanguin, or cinnabarinic acid in the fruiting bodies.[72,73] Phenoxazines can be synthesized by heating 2-aminophenol and 2-aminophenol hydrochloride in an equimolar mixture.[74] Other methods comprise, e.g., potassium or cesium carbonate with copper in organic solvents.[75,76] The phenazine pyocyanin is one of the pigments that is produced by most of the strains of the pathogenic bacterium *Pseudomonas aeruginosa*.[77] The chemical production of phenazines may be accomplished, among others, by the Wohl−Aue reaction, which includes heating upon 200 °C, or the Beirut reaction. Transition metals may be used as catalysts similar to the syntheses of phenothiazines and phenoxazines (for reviews, see Laursen and Nielsen[78] or Chaudhary and Khurana[79]).

We developed a one-pot reaction with a nonstoichiometric and environmentally friendly catalyst, which can be performed in an aqueous solution (with less than 5% methanol, which can be easily replaced by solvents such as ethanol[80] due to similar characteristics) at room temperature. The reactions were performed in the presence of oxygen, at atmospheric pressure, and at a moderate pH value (pH 5). These characteristics of the introduced laccase-mediated reactions are valuable for the green synthesis of cyclic products as well as dimers and trimers. The use of reaction partners with amino, hydroxyl, and/or thiol groups presented the possibility to form new heterocycles bio-enzymatically.

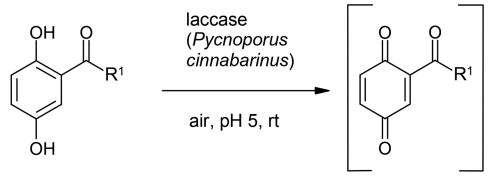
RESULTS

General Observations. The thiols and amines used in this study consist of a benzene ring with two functional groups in *ortho*-position to each other. The compounds contain a thiol group and/or amino group. Hydroxyl, amino, methyl, or nitro groups can be second substituents. The thiols and amines were incubated with 2,5-dihydroxybenzoic acid or with one of its derivatives and the laccase of *Pycnoporus cinnabarinus*. Oxidative C−N bond formation followed by cyclization was the predominant reaction, resulting in phenothiazines, phenoxazines, and phenazines.

All reactions were performed with 0.5 U laccase of *P. cinnabarinus* as the catalyst in sodium acetate buffer (pH 5) at room temperature with a reactant concentration of 1 mM. The reactions were analyzed in the course of an incubation time of 24 h.

Reactions of 2,5-Dihydroxybenzoic Acid and Its Derivatives. The laccase-mediated reactions of 2,5-dihydroxybenzoic acid (**1a**), 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (**1b**), and 2,5-dihydroxybenzoic acid methylester (**1c**) may result in the formation of the respective benzoquinone and/or a radical (**2**, Scheme 1).[81] These

Scheme 1. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c for the Synthesis of the Respective Benzoquinone (2) Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



1a R^1 = OH
1b R^1 = NHC_2H_4OH
1c R^1 = OCH_3

2

intermediates can be attacked by water or solvents such as methanol, resulting in hydroxylated and methoxylated benzoquinonoid products.[81,82] These side reactions may diminish the yield of products formed with additional coupling partners such as thiols and amines.

Under the chosen reaction conditions, none of these products were formed in controls (without laccase).

Reactions of 2-Aminothiophenol with 2,5-Dihydroxybenzoic Acid. The reactions of **1a−1c** with 2-aminothiophenol (**3a**) resulted in laccase-mediated formation of adducts (**4a−4c**) and phenothiazines (**5a−5c**, Scheme 2 and Table 1). The yields of the phenothiazines ranged from 5% (**5c**) to 29% (**5b**).

Under the chosen reaction conditions, none of these products were formed in controls (without laccase).

The products **4a−4c** were not stable and cannot be isolated under the used conditions. Nevertheless, the recorded mass spectra of the reaction assays allowed the identification of the products (Figures S6, S8, S10, S12, Supporting Information).

The incubation of **3a** resulted in the formation of a disulfide (**6**, Scheme 3).

Retention time, UV−vis maxima, and LC/MS analyses in comparison with the commercially available reference 2-aminophenyl disulfide confirmed the proposed dimeric structure for **6** (Table S1, Supporting Information for product 6: R_f (HPLC) 11.54 min; UV−vis (MeOH) λ_{max} 215, 334 nm; LC/MS *m/z* (rel. intensity) AP-ESI pos. mode $[M + H]^+$ 249.0 (100); Table S2, Supporting Information for reference 2-aminophenyl disulfide: R_f (HPLC) 12.02 min, UV−vis (MeOH) λ_{max} 218, 335 nm; LC/MS *m/z* (rel. intensity) AP-ESI pos. mode $[M + H]^+$ 249.0 (100)). The two molecules of **3a** are connected via a S−S bond.

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Scheme 2. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c with 2-Aminothiophenol 3a for the Synthesis of Adducts 4a−4c and the Cyclization Products 4a-Hydroxy-2-oxo-phenothiazines 5a−5c using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)

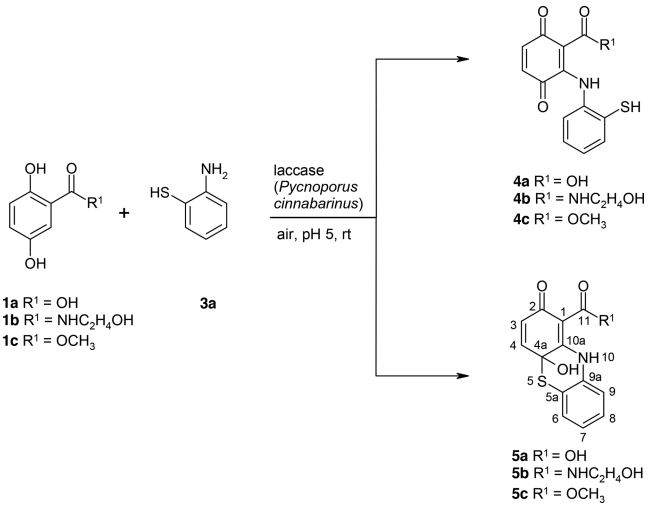
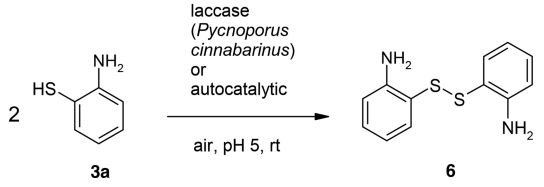


Table 1. Reactions of 1a−1c with 3a and Formed Products Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5) within an Incubation Time of 24 h (Reactant Concentration, 1 mM)

reactant 1	R^1	reactant 3	product	yield[a]
1a	OH	3a	4a	-
			5a	-
			7a	-
			9a	-
			10a	-
1b	NHC_2H_4OH	3a	4b	
			5b	29%
			7b	-
			9b	6%
			10b	
1c	OCH_3	3a	4c	-
			5c	5%
			7c, 8c	-, 5%
			9c	-
			10c	

[a]-, not determined; no isolation of products was performed.

Scheme 3. Laccase-Catalyzed or Autocatalytic Reaction of 2-Aminothiophenol (3a) for the Synthesis of the Disulfide 6 Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



The laccase-mediated reaction of **1a−1c** and **6** resulted in the formation of **7a−7c** (Scheme 4).

Products **7a−7c** were not stable and cannot be isolated under the used conditions. Nevertheless, the recorded mass spectra of the reaction assays allowed the identification of the products (Figures S33, S35, S37, S39, S40 and Tables S11−S14, Supporting Information). In addition, the NMR data of **8c** in the mixture with **5c** support the structure of [2-[(2-aminophenyl)disulfanyl]anilino]-3,6-dihydroxy-benzoic acid.

The reactions of **1b** and **3a** or 2-aminophenyl disulfide (reference for product **6**) were chosen to determine the laccase-mediated reaction in more detail: The reaction of **1b** and **3a** resulted in the formation of **6**, **4b**, **5b**, and **7b**. The amount of product **5b** increased with concomitant decrease of products **4b** and **7b** (Figure 1). Products **4b** and **7b** peaked after 20 and 10 min, respectively, and decreased afterward, whereas the product **5b** was detected after 20 min and increased rapidly up to 100 min. Afterward, **5b** was stable within 24 h (data not shown).

This supports the assumption that products **4b** and/or **7b** are possible reactants for the formation of **5b** (Scheme 5).

The laccase-mediated reaction of **1b** with 2-aminophenyl disulfide (reference for product **6**) resulted also in the formation of products **4b**, **5b**, and **7b** in similar product yields. The formation of products **4b** and **5b** require the cleavage of 2-aminophenyl disulfide or the 2-aminophenyl disulfide part in **7b** (Scheme 5), but no **3a** was detected in the homomolecular reaction of 2-aminophenyl disulfide. Nevertheless, in the heteromolecular reactions of **1b** with **3a** or **1b** and 2-aminophenyl disulfide, a product with a retention time of 5.57 min was detected, which possessed a similar UV−vis spectrum as **3a**. Additionally, the retention time of the product was only 1 min later compared with **3a** (R_f (HPLC) 4.59 min). The concurrent formation of this product with the increase of product **5b** may explain the synthesis of **4b** and **5b** in the reaction of **1b** and 2-aminophenyl disulfide.

The disulfide **6** was detected in the reactions without addition of laccase but none of the further formed heteromolecular products.

A parallel pathway can be hypothesized for the reaction of **1a−1c** with **3a** because of the additional formation of products **9a−9c** and **10a−10c** (in parallel to products **4**, **5**, **7**, and **8**). For the formation of products **9** and **10**, the amino group of **3a** reacted with the C-2 atom of the aromatic ring of **1a−1c** followed by a ring closure via the thiol group on the C-3 atom of the aromatic ring of **1a−1c**. In contrast to that reaction, the formation of products **4**, **5**, **7**, and **8** started with an attack of the amino group of **3a** at the C-6 atom of the aromatic ring of **1a−1c**. The attack at the C-6 atom of **1a−1c** resulted in the formation of adducts **4a−4c**, **7a−7c**, and **8c** and the cyclization products 4a-hydroxy-2-oxo-phenothiazines **5a−5c**, whereas the attack at the C-2 atom of **1a−1c** resulted in the formation of 3-hydroxy-phenothiazines **9a−9c**. The product **9b** was not detected in the reaction of **1b** and 2-aminophenyl disulfide (**6(ref)**).

In the course of the LC/MS analyses, phenothiazines **9a−9c** and **10a−10c** were detected (Scheme 6).

The LC/MS spectra contain both masses for 3-oxo-phenothiazines and 3-hydroxy-10*H*-phenothiazines (such as for **9b**: *m*/*z* (rel. intensity) AP-ESI pos. mode $[M + H]^+$, 303.0 (5); for **10b**: *m*/*z* (rel. intensity) AP-ESI pos. mode $[M + H]^+$, 301.0 (100); Figure S44 and Table S16) but in very low intensity for 3-hydroxy-phenothiazines (**9a−9c**).

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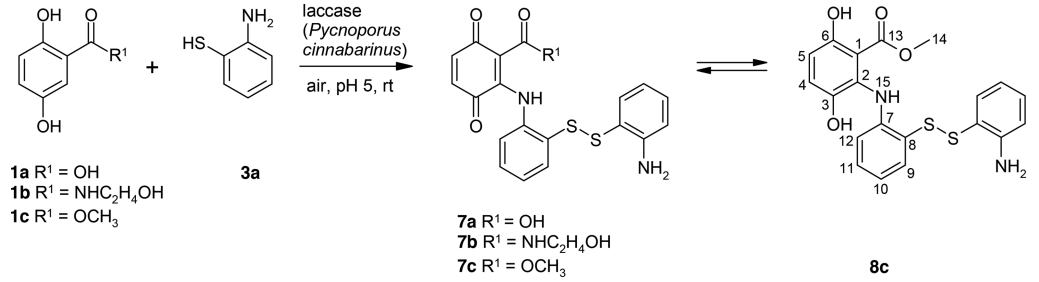
Scheme 4. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c with 2-Aminothiophenol 3a for the Synthesis of Adducts 7a−7c and 8c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



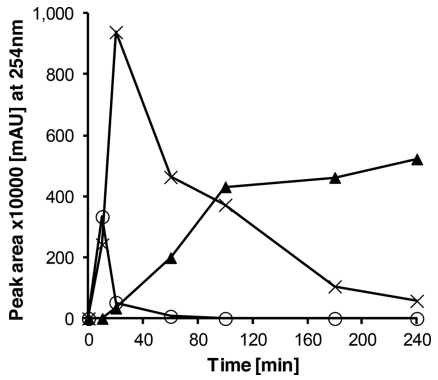


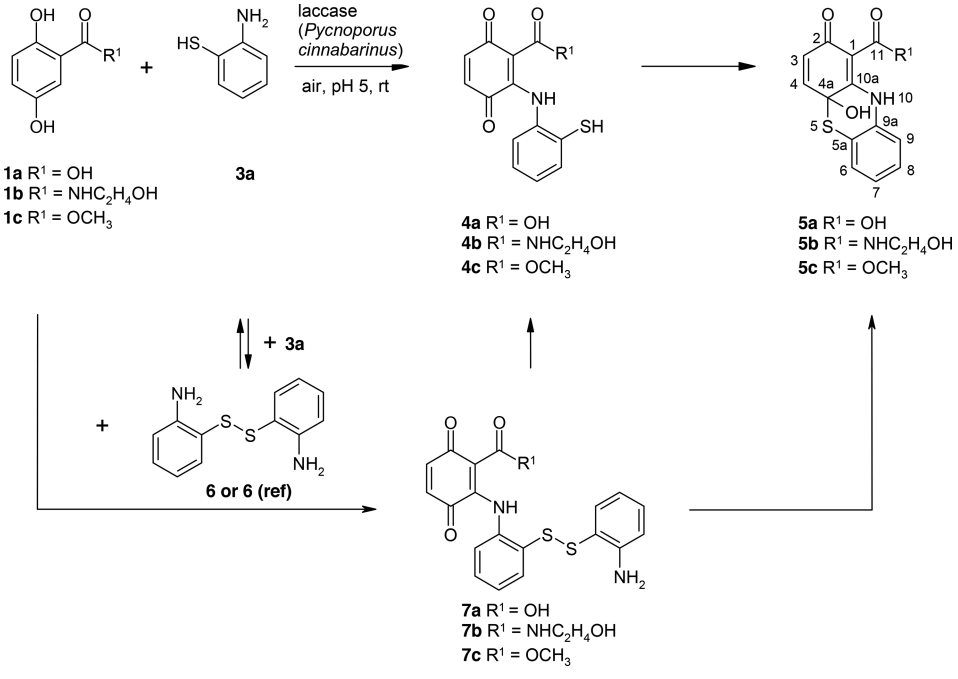
Figure 1. Reaction course of product formation for **4b** (cross), **5b** (filled triangle), and **7b** (open circle) at equimolar concentrations (1 mM) of reactants 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide (**1b**) and 2-aminothiophenol (**3a**) using 0.5 U laccase of *P. cinnabarinus* as the catalyst in sodium acetate buffer (pH 5; reactant concentration, 1 mM).

The NMR data of **9b** clearly identify this product as 3-hydroxy-*N*-(2-hydroxyethyl)-10*H*-phenothiazine-1-carboxamide (Figures S44-S48 and Table S16), which may be easily transformed via oxidation to the respective 3-oxo-phenothiazine.

Reactions of 2-Aminophenol or *ortho*-Phenylenediamine with 2,5-Dihydroxybenzoic Acid Derivatives. In the laccase-catalyzed reaction of **1a−1c** and 2-aminophenol (**3b**), the phenoxazines (**11a−11c**) were formed (Scheme 7 and Table 2). At first, amination in the *ortho*-position to the side chain—in the case of the product **11b** in the *ortho*-position of the amide group—took place with subsequent bond formation between the hydroxyl group of **3b** and C-5 of the aromatic ring.

The reactions of **1a−1c** and *ortho*-phenylenediamine (**3c**) resulted in the formation of phenazines (**12a−12c**; Scheme 8) due to the two neighboring amino groups of **3c**. The reaction of **3c** and **1b** resulted in almost one heteromolecular product, whereas for the reaction with **1c**, at least two main products were detected. This may explain the small yield of **12c** in

Scheme 5. Laccase-Catalyzed Reactions of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c with 2-Aminothiophenol 3a, with Its Dimer 6 or with the Commercially Available Reference 2-Aminophenyl Disulfide (6(ref)) for the Synthesis of Adducts 4a−4c and 7a−7c and the Cyclization Products 4a-Hydroxy-2-oxo-phenothiazines 5a−5c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



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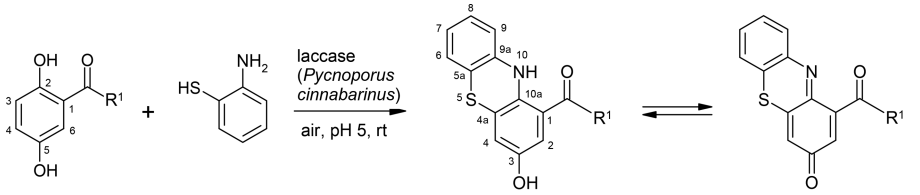
Scheme 6. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c and 2-Aminothiophenol 3a for the Synthesis of 3-Hydroxy-10*H*-phenothiazines 9a−9c and 3-Oxo-phenothiazines 10a−10c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



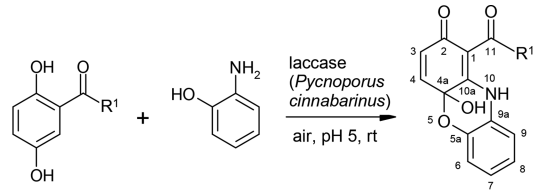
1a R^1 = OH
1b R^1 = NHC_2H_4OH
1c R^1 = OCH_3

3a

9a R^1 = OH
9b R^1 = NHC_2H_4OH
9c R^1 = OCH_3

10a R^1 = OH
10b R^1 = NHC_2H_4OH
10c R^1 = OCH_3

Scheme 7. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c and 2-Aminophenol 3b for the Synthesis of Phenoxazines 11a−11c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)

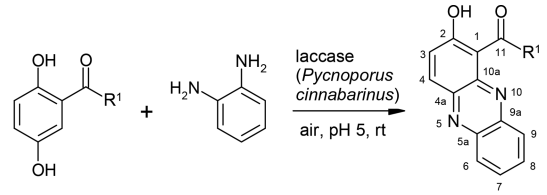


1a R^1 = OH
1b R^1 = NHC_2H_4OH
1c R^1 = OCH_3

3b

11a R^1 = OH
11b R^1 = NHC_2H_4OH
11c R^1 = OCH_3

Scheme 8. Laccase-Catalyzed Reaction of 2,5-Dihydroxybenzoic Acid Derivatives 1a−1c and *ortho*-Phenylenediamine 3c for the Synthesis of Phenazines 12a−12c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



1a R^1 = OH
1b R^1 = NHC_2H_4OH
1c R^1 = OCH_3

3c

12a R^1 = OH
12b R^1 = NHC_2H_4OH
12c R^1 = OCH_3

Table 2. Reactions of 1a−1c with 3b−3f and Formed Products Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5) within an Incubation Time of 24 h (Reactant Concentration, 1 mM)

reactant **1**	R^1	reactant **3**	product	yield[a]
1a	OH	**3b**	**11a**	-
		3c	**12a**	-
1b	NHC_2H_4OH	**3b**	**11b**	8%
		3c	**12b**	71%
		3d	**13a**	15%
			14a	15%
			15a	-
		3e	**13b**	16%
			14b	
			15b	-
		3f	**13c**	-
			15c	-
1c	OCH_3	**3b**	**11c**	-
		3c	**12c**	7%

[a]-, not determined; no isolation of products was performed.

comparison with **12b**. (We were unable to isolate the second product of the reaction of **3c** with **1c**.)

In the course of the reactions of **3b** or **3c** with a second reactant and laccase, for **3b** and **3c**, also ring-closure mechanisms were detected. These reactions resulted in phenazines and phenoxazinones that were not further characterized due to only limited influence on the product formation with **1a−1c**. Such phenazines and phenoxazinones formed from *ortho*-diamines and *ortho*-aminophenols with laccase were also described previously.[83,84]

Reactions of 2-Methylaniline, 2-Nitroaniline, or 2-Methylbenzenethiol with 2,5-Dihydroxybenzoic Acid Derivatives. Although all laccase-mediated reactions of **3a−3c** resulted in the formation of cyclic products, we determined no cyclization between 2-methylaniline (**3d**), 2-nitroaniline (**3e**), or 2-methylbenzenethiol (**3f**) and **1b**, respectively. In these reactions, different adducts were detected (Scheme 9). The adduct of the reaction of **1b** and **3d** was isolated as a mixture of the quinonoid (**13a**) and hydroquinonoid (**14a**) forms, which were also described previously for the reaction with 2-aminobenzoic acid.[37] Similarly, **14b** was described by NMR analyses, whereas **13b** was only detected by LC/MS. For the formation of **13c−15c**, a C−S bond was assumed.

Detailed Structural Characterization of Products. The various cyclic products possessed different UV−vis spectra. The phenothiazines (**5a−5c**) and phenoxazines (**11a−11c**) had an orange-brown color, whereas the phenazines (**12a−12c**) were red or yellow. The phenothiazine **9**/**10b** was violet. Phenothiazines **5a−5c** were characterized by three maxima under 300 nm and one intense maximum around 390 nm, whereas phenothiazines **9**/**10a−10c** had an intense maximum around 240 nm, two to three maxima under 400 nm, and another one around 500 nm. The phenoxazines (**11a−11c**) had two maxima under 300 nm and one intense maximum around 380 nm. The phenazines (**12a−12c**) possessed one intense maximum around 250 nm and two maxima above 360 nm, which is in line with the literature.[77]

In the laccase-mediated reaction of **1b** and **3a**, the phenothiazine **5b** was isolated for mass spectroscopy (LC/MS *m*/*z* AP-ESI pos. mode $[M + H]^+$, 319.0 (100); neg. mode $[M - H]^-$, 316.9 (100)). These mass data can be attributed to the amination of **3a** on the quinonoid form of **1b** and

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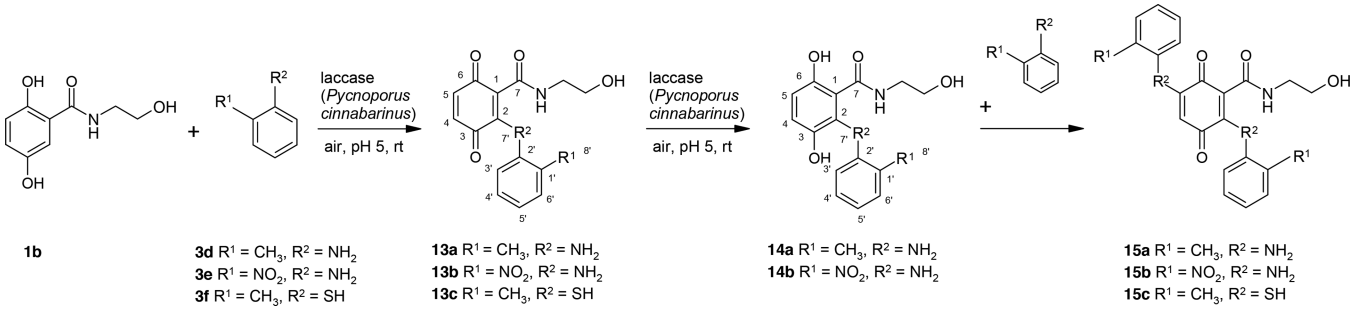
Scheme 9. Laccase-Catalyzed Reaction of 2,5-Dihydroxy-*N*-(2-hydroxyethyl)benzamide 1b and 2-Methylaniline 3d, 2-Nitroaniline 3e, or 2-Methylbenzenethiol 3f for the Synthesis of Quinonoid 13a−13c or Hydroquinonoid 14a and 14b and Adducts 15a−15c Using 0.5 U Laccase of *P. cinnabarinus* as the Catalyst in Sodium Acetate Buffer (pH 5; Reactant Concentration, 1 mM)



cyclization via the thiol group of **3a** on the carbonyl group of the quinone (C-5 in the reactant and C-4a in the product), resulting in a six-membered nonaromatic ring. The NMR analyses confirmed the formation of two bonds between **1b** and **3a** (Figure 2).

Multiplicity of H-4 and H-3 suggested that the amination step took place at C-10a. The HMBC correlations (Figure 2) of the proton H-10 unambiguously identified this amination at C-10a. A signal for the hydroxyl group at C-4a (7.53 ppm) was observed. This signal of **5b** was a broad signal without any HMBC correlations, but this signal of **5c** (7.50 ppm) showed HMBC correlations to C-4a (69.7 ppm) and C-10a (154.6 ppm), supporting the concept of cyclization of the thiol group at C-4a and the removal of the *para*-quinonoid character. The chemical shift to a higher field (69.6 ppm) of the C-4a of **5b** resulted in the assumption of a nonaromatic, noncarbonyl carbon atom. Additionally, ^{13}C NMR showed only one typical signal for quinones in the range of 180 ppm, indicating only one quinonoid carbonyl group for **5b**. The chemical shifts within the transformed structure of **1b** and **1c** are similar to previously described structures.[37] The analyses and the comparison led to the identification of **5b** as 4a-hydroxy-*N*-(2-hydroxyethyl)-2-oxo-10*H*-phenothiazine-1-carboxamid.

Products **9b** and **10b** could only be isolated as a mixture with MS data AP-ESI in positive mode for the oxo structure and with NMR data for the hydroxyl structure. The molecular mass of 300 was attributed to the amination of **3a** on the quinonoid form of **1b** and cyclization via the thiol group of **3a**, resulting in **10b**. The postulated product structure of **9b** was confirmed by NMR. ^{1}H NMR spectral data of **9b** showed characteristic signals for both reactants (Figure 3). Multiplicity of H-2 and H-4 suggested that the amination step took place at C-10a. The HMBC correlations (Figure 3) of the proton H-10 clearly confirmed this amination at C-10a. The HMBC spectrum showed also correlations between protons H-2 and H-4 and the aminated C-10a atom in the typical range of 130−150 ppm and between protons H-2 and H-4 and the hydroxylated C-3 atom (152.4 ppm), unambiguously showing **9b** to be a nonquinonoid aminated and hydroxylated structure substituted at C-10a and C-4a. All MS and NMR results led to the identification of **10b** as *N*-(2-hydroxyethyl)-3-oxo-phenothiazine-1-carboxamide and **9b** as 3-hydroxy-*N*-(2-hydroxyethyl)-10*H*-phenothiazine-1-carboxamide.

In the laccase-mediated reaction of **1b** and **3b**, the phenoxazine **11b** was isolated. The mass spectroscopy data (LC/MS *m*/*z* AP-ESI pos. mode $[M + H]^+$, 303.0 (100)) confirmed the formation of an adduct similar to that described above for **5b**. The mass data can also be attributed to amination of **3b** on the quinonoid form of **1b**, but the cyclization took place via the hydroxyl group of **3b**, yielding **11b**. The carbonyl group (C-4a) of the quinone intermediate of **1b** is the C-atom for cyclization for both **5b** and **11b**, resulting in six-membered nonaromatic rings with an ether bridge in the structure of **11b** but with a thioether bridge for **5b**. The NMR analyses of **11b** also confirmed the formation of two bonds between **1b** and **3b** (Figure 4) as described for **5b**. The HMBC correlations (Figure 4) of the proton H-10 also supported the amination at C-10a for **11b**. The signal for the hydroxyl group at C-4a (8.20 ppm) showed HMBC correlations to C-4a (86.8 ppm), C-4 (137.5 ppm), and C-10a (156.1 ppm) and ^{1}H-^{1}H COSY correlations to H-3 (6.22 ppm), H-4 (6.92 ppm), and H-9 (7.43 ppm), supporting the concept of cyclization of the hydroxyl group of **3b** at C-4a and the removal of the *para*-quinonoid character. The chemical shift to a higher field (86.8 ppm) of the C-4a of **11b** resulted also in the assumption of a nonaromatic, noncarbonyl carbon atom. Additionally, as described for **5b**, ^{13}C NMR showed only one typical signal for quinones at 180 ppm, indicating only one quinonoid carbonyl group and the structure of 4a-hydroxy-*N*-(2-hydroxyethyl)-2-oxo-10*H*-phenoxazine-1-carboxamid for **11b**.

DISCUSSION

The structural characterization of the products by MS and NMR analyses together with UV−vis data led to the description of cyclic products as well as different additional products (Scheme 10). The type of product was dependent on the coupling partners and the amount of reactive groups. Thus, the homomolecular reaction of **3a** resulted in the formation of a disulfide (**6**). Heteromolecular products **4a−4c** and disulfides **7a−7c** and **8c** were formed in the reactions of **3a** with **1a−1c**, whereby the disulfide **6** is part of adducts **7a−7c** and **8c**. The reactions of **1a−1c** and coupling partners with one amino group and an adjacent thiol group (**3a**) or, instead, a hydroxyl group (**3b**) as well as substances with two amino groups (**3c**) resulted in cyclic products such as phenothiazines (**5a−5c** and **9**/**10a−10c**), phenoxazines (**11a−11c**), or phenazines (**12a−12c**). Compounds with only one reactive group (**3d−3f**) for the laccase-mediated heteromolecular coupling resulted in quinonoid (**13a−13c**, and **15a−15c**) and hydroquinonoid (**14a**, **14b**) products. To the best of our knowledge, this is the first description of cyclic products **5a−5c** and **9**/**10a−10c** for laccase-mediated reactions.

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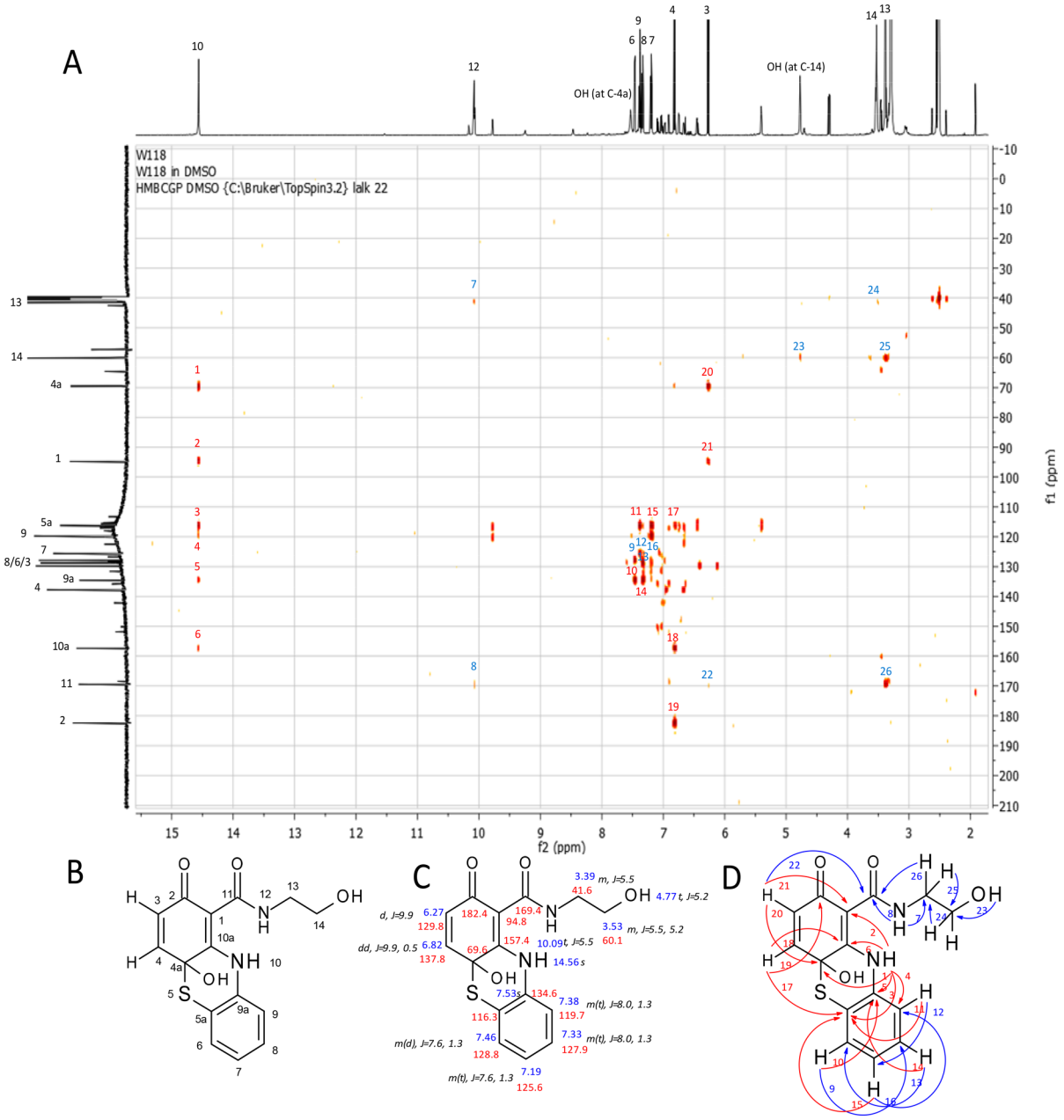

Figure 2. Product **5b**: (A) HMBC spectrum of product **5b** with HMBC correlation numbers: red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds. (B) Numbering of C-atoms. (C) ^{1}H (blue) and ^{13}C (red) assignments (chemical shifts are expressed in *d* (ppm) calibrated on the resonances of the residual nondeuterated solvent DMSO) and multiplicity of the ^{1}H signals and *J* values (black). *J* values are in Hz. (D) HMBC correlations (H → C): red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds.

All heteromolecular reactions started with the laccase-mediated oxidation of the respective 2,5-dihydroxybenzoic acid derivative, which resulted in the formation of quinonoid derivatives (**2**).

Two reaction pathways for the formation of different phenothiazines were described. The main pathway for products **5a**−**5c** comprised the intermolecular Michael addition (1,4-addition) of **3a** via the amino group on the quinonoid derivative **2** of **1a**−**1c**. After the second oxidation, the heteromolecular products (**4a**−**4c**) were formed, which undergo intramolecular 1,2-addition. Similar reactions were described previously for 2,5-dihydroxybenzoic acid derivatives and five- or six-membered amines containing an amino group and carboxamide group.[37] The key difference of the present research results is that C−S and C−N bonds were formed within one single product, whereas the bond formation of C−O and C−N bonds has been described until now. Another possibility is the dimerization of **3a**, which is the prerequisite for the reaction with **1a**−**1c** forming the disulfides **7a**−**7c**, which may react to the phenothiazines (**5a**−**5c**) or additional products (**4a**−**4c**). In this case, a laccase-mediated bond cleavage may be conceivable, similar to the previously described cleavage processes for amino compounds.[12] The second pathway for phenothiazine synthesis (**9a**−**9c** and **10a**−**10c**) started with amination of **3a** on the C-2 atom of the aromatic ring of **1a**−**1c** with subsequent C−S bond formation

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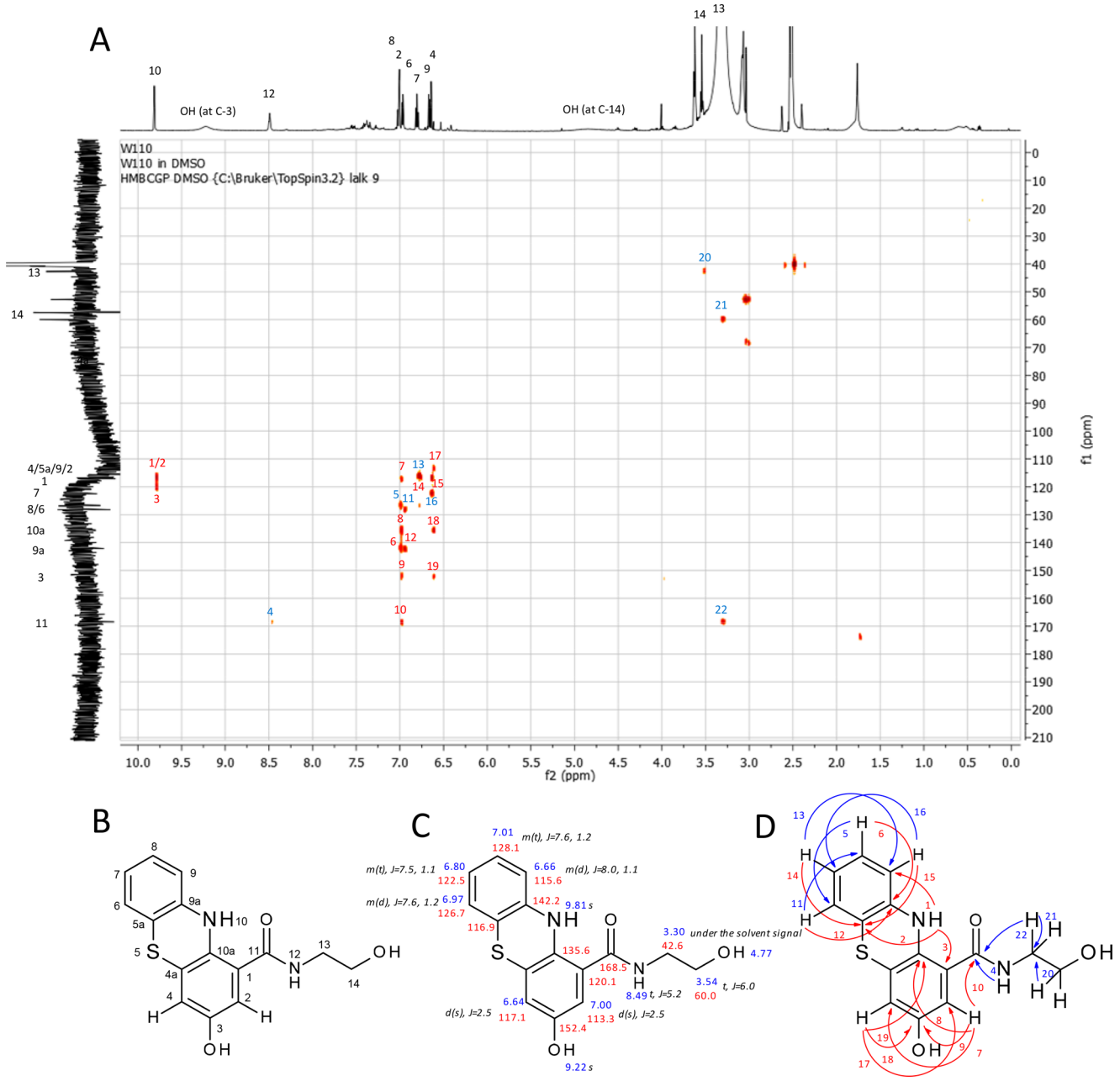

Figure 3. Product **9b**: (A) HMBC spectrum of product **9b** with HMBC correlation numbers: red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds. (B) Numbering of C-atoms. (C) ^{1}H (blue) and ^{13}C (red) assignments (chemical shifts are expressed in *d* (ppm) calibrated on the resonances of the residual nondeuterated solvent DMSO) and multiplicity of the ^{1}H signals and *J* values (black). *J* values are in Hz. (D) HMBC correlations (H → C): red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds.

via the thiol group and the aromatic ring of **1a−1c**. But we were unable to rule out the possibility that the thiolation on the C-3 atom of the aromatic ring of **1a−1c** took place first and afterward the amination. However, compared to all of our previous described reaction pattern, this is the first time that we detected bond formations on C-2 and C-3 atoms of the laccase substrate 2,5-dihydroxybenzoic acid and its derivatives (**1a−1c**). A plausible explanation is based on the different nature of the thiol group compared to the amino and hydroxyl groups.

However, it must be taken into account that the *p*-hydroquinone substrates are very different from 2,5-dihydroxybenzoic acid and its derivatives. Thus, a similar pathway was described for the laccase-mediated reactions of *p*-hydroquinone or its derivatives with 5-substituted-4-amino-3-mercapto-1,2,4-triazoles or 2-amino-thiophenol described by Bhalerao et al.[7] and Cannatelli and Ragauskas,[10] respectively. In the case of **9a−9c** and **10a−10c**, the reaction of the thiol group of the reaction partner took place on the carbonyl group of the quinonoid form of **1a−1c** and the reaction of the amino group on the adjacent unsubstituted CH group, whereas Cannatelli and Ragauskas[10] described the reaction of the amino group on the carbonyl group of the quinonoid form of the laccase substrates and the reaction of the thiol group on the adjacent unsubstituted CH group.

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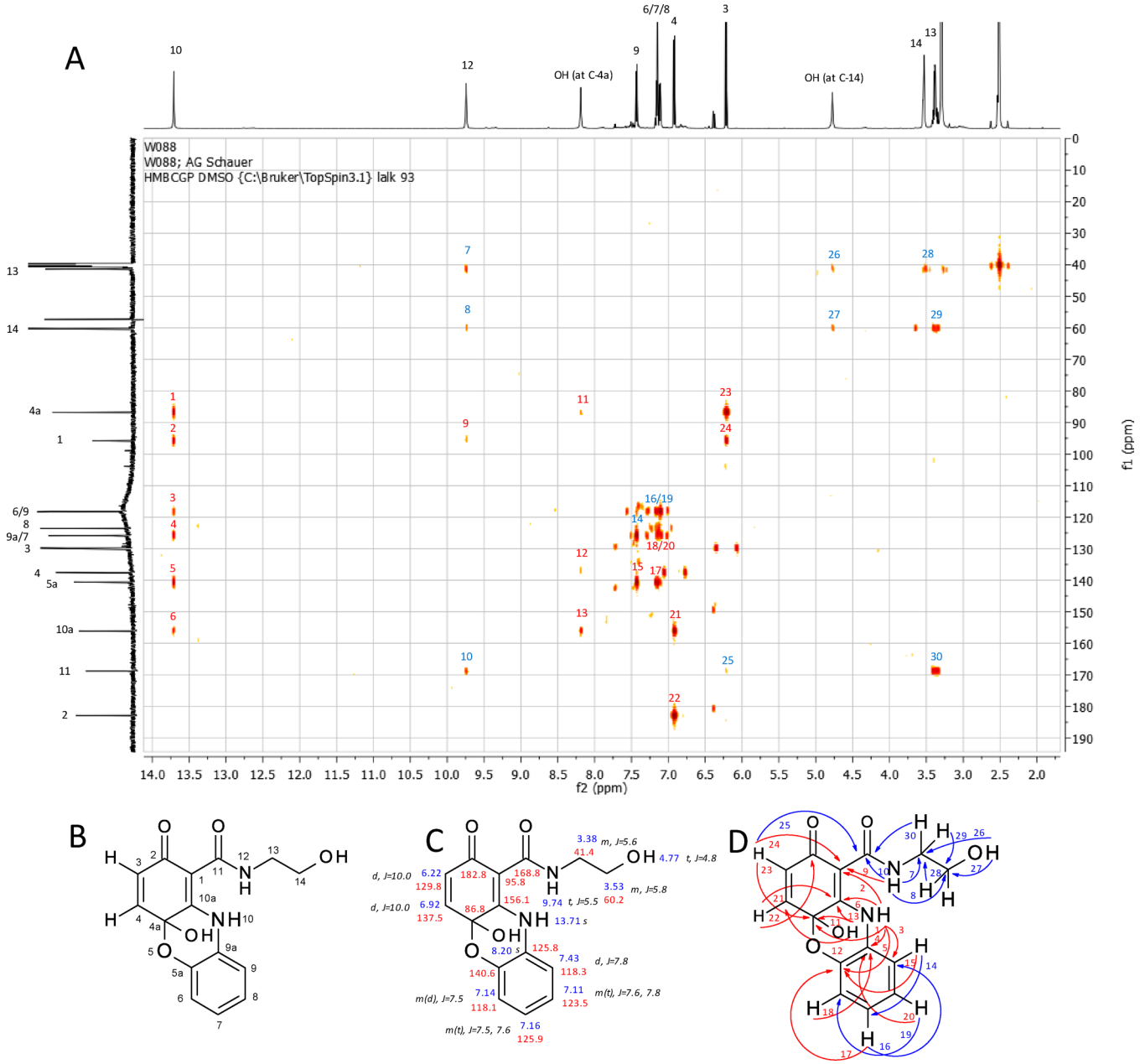

Figure 4. Product **11b**: (A) HMBC spectrum of product **11b** with HMBC correlation numbers: red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds. (B) Numbering of C-atoms. (C) ^{1}H (blue) and ^{13}C (red) assignments (chemical shifts are expressed in *d* (ppm) calibrated on the resonances of the residual nondeuterated solvent DMSO) and multiplicity of the ^{1}H signals and *J* values (black). *J* values are in Hz. (D) HMBC correlations (H → C): red, correlations that confirm the novel structure; blue, correlations that are similar to that of the parent compounds.

The authors proposed an addition−reaction of the amino group with the C-atom at the carbonyl group of the quinone, resulting in a quinonimine, with subsequent reaction of the thiol group with the adjacent C-atom of the benzene ring. Bhalerao et al.[7] described the reaction of *p*-hydroquinone with 5-substituted-4-amino-3-mercapto-1,2,4-triazoles. The coupling partners were connected via C−S and C═N bonds in the formed benzothiadiazinones. Again, a quinonimine was formed due to the reaction of the amino group with the C-atom at the keto group of the quinone.

Bhalerao et al.[7] as well as Cannatelli and Ragauskas[10] used *p*-hydroquinone or its derivatives. This may explain the different reaction pathway for the herein used 2,5-dihydroxybenzoic acid derivatives.

The formation of products **7a−7c** was supported by the NMR analyses of **5c**. The disulfide (**6**) of **3a** was described previously by Cannatelli and Ragauskas.[10] For laccase-mediated reactions, disulfides **7a−7c** are unusual. In most cases, the reaction of two amino compounds with dihydroxybenzoic acid derivatives proceeded via two C−N bonds in the *para*-position.[32] The UV−vis spectra of products **7a−7c** (**7a**: 210, 455 nm; **7b**: 209, 492 nm; **7c**: 210, 473 nm) resembled more monoaminated quinonamines with at least one absorption spectrum under 300 nm and one around 500 nm than diaminated quinonamines with at least two maxima under

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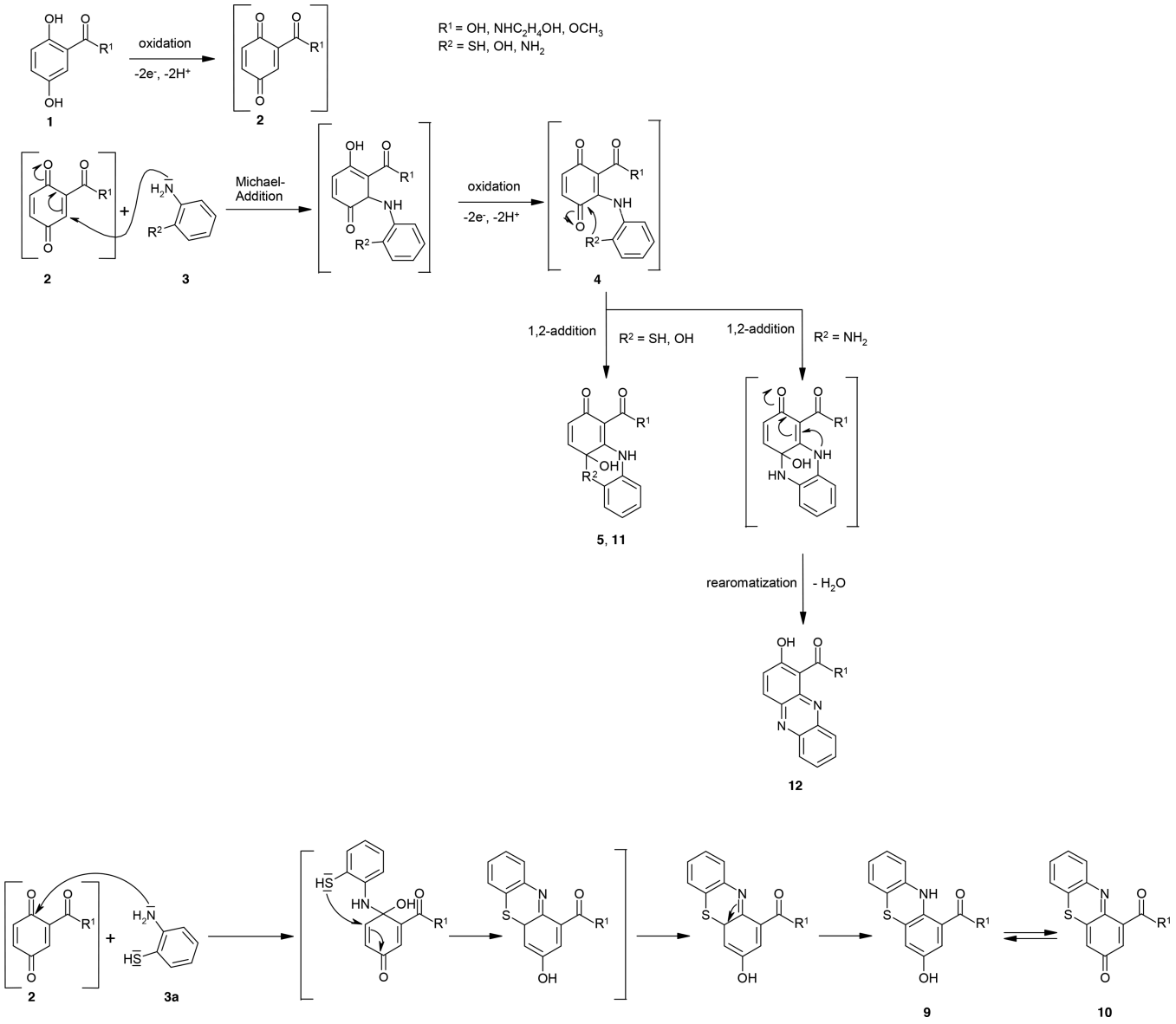
Scheme 10. Possible Reaction Mechanisms



400 nm.[12,25,85] These absorption maxima were not only in line with those for the mono- and diaminated products (**13a**, **13b**, and **15a**, **15b**) but also those for the monothiolated product **13c** and dithiolated product **15c**. These products were formed by nucleophilic addition of one or two molecules of **3f** with **1b**, forming C−S bonds. In this case, the thiol groups and not the amino groups (as for **13a**, **13b**, **15a**, and **15b**) were the donors in the Michael addition.[86] The reaction of *para*-hydroquinones and thiols resulting in different adducts was described previously.[11,36,87] Cyclization involving two SH groups, which resulted in 2,3-ethylenedithio-1,4-quinones with yields of 37−74%, was also described.[38]

In summary, the cyclic products described in our study were isolated with yields of 4% up to 68%. This confirms the complexity, in comparison to the so-called "easy" and "straightforward" laccase-catalyzed reactions of 2,5-dihydroxybenzoic acid derivatives and reaction partners with only one amino group,[24,26−29] of such cyclization reactions accompanied by simultaneous formation of homomolecular products of the amines and by-products, which diminished the yields. Further studies will lead to strategies for a higher product yield through variations of reaction parameters such as the kind of laccase, pH value of the reaction assay, or concentration of reactants.

CONCLUSIONS

The introduced one-pot laccase-mediated reactions of cyclic products as well as additional products are consistent with the principles of green chemistry defined by Anastas and Warner.[88] The utilization of laccases as nonstoichiometric catalysts in an aqueous solution with less than 5% solvent (which is only necessary for the solubilization of the reagents and can be replaced in most cases by water) and the room temperature make this process an attractive alternative to chemical syntheses.

EXPERIMENTAL SECTION

Enzymes. The used laccase was obtained from *P. cinnabarinus* SBUG-M 1044. The white rot fungus was isolated from an oak tree in northern Germany and is deposited at the

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strain collection of the Department of Biology of the University of Greifswald (SBUG).

Cultivation of *P. cinnabarinus* SBUG-M 1044 and crude preparation of laccase were carried out as we reported previously.[19] This enzyme preparation contains only isoenzymes of laccase but no other enzymes and was used always in 20 mM sodium acetate buffer (SAB; pH 5) because of the pH optimum around pH 5.[19,89]

Measurement of Laccase Activity. The activity of laccase was determined spectrophotometrically at 420 nm with ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) as the substrate[90] using the method described by Jonas et al.[19] One unit (1 U) is defined as 1 μmol·mL^{-1}·min^{-1}.

Experimental Procedures. *Analytical Procedure.* For analytical experiments, amines, thiols (1 mM), and the respective dihydroxylated compound (1 mM) were incubated with laccase (activity, 0.5 U). Reaction mixtures were incubated with agitation at 200 rpm at room temperature in the dark. The reaction mixtures were analyzed by HPLC. The separation of the substances was achieved with a RP18 column at a flow rate of 1 mL/min. A solvent system consisting of methanol (eluent A) and 0.1% phosphoric acid (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% methanol within 14 min, was used. Controls were performed without the addition of laccase.

Product Isolation. All reaction mixtures for product isolation were performed with laccase of *P. cinnabarinus* (final activity, 0.5 U) in SAB. Isolation steps were performed by solid-phase extraction with a RP18 silica gel column (60 mL, 10 g of adsorbent material, Phenomenex, Strata, Germany). The product **5b** was isolated from the reaction mixture (320 mL) after an incubation period of 24 h (**1b**:**3a**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 20 mL of methanol/distilled water (20:80, v/v) and 40 mL of methanol/distilled water (40:60, v/v) were used to remove undesired impurities. Elution of the orange fraction was performed with 30 mL of methanol. The product **5c** (mixture with **8c**) was isolated from the reaction mixture (400 mL) after an incubation period of 4.5 h (**1c**:**3a**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 30 mL of methanol/distilled water (20:80, v/v) and 30 mL of methanol/distilled water (40:60, v/v) were used to remove undesired impurities. Elution of the orange fraction was performed with 30 mL of methanol.

The product **9**/**10b** was isolated from the reaction mixture (320 mL) after an incubation period of 20 min (**1b**:**3a**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture and washing steps with 20 mL of methanol/distilled water (20:80, v/v) and 40 mL of methanol/distilled water (50:50, v/v), the product was eluted with 20 mL of methanol/distilled water (50:50 v/v). The product **11b** was isolated from the reaction mixture (280 mL) after an incubation period of 4 h (**1b**:**3b**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 40 mL of methanol/distilled water (40:60, v/v), 15 mL of methanol/ distilled water (60:40, v/v), and 5 mL of methanol/distilled water (80:20, v/v) were used to remove undesired impurities. The product was eluted with additional 10 mL of methanol/distilled water (80:20, v/v). The product **12b** was isolated from the reaction mixture (240 mL) after an incubation period of 20 min (**1b**:**3c**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 20 mL of methanol/distilled water (20:80, v/v) and 50 mL of methanol/distilled water (60:40, v/v) were used to remove undesired impurities. The product was eluted with 25 mL of methanol. The product **12c** was isolated from the reaction mixture (520 mL) after an incubation period of 3.5 h (**1c**:**3c**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 40 mL of methanol/distilled water (40:60, v/v), 15 mL of methanol/distilled water (60:40, v/v), 5 mL of methanol/distilled water (80:20, v/v), and 10 mL of methanol/distilled water (80:20, v/v) were used to remove undesired impurities. The product was eluted with additional 20 mL of methanol/distilled water (80:20, v/v). The product **13**/**14a** was isolated from the reaction mixture (120 mL) after an incubation period of 2 h (**1b**:**3d**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 20 mL of distilled water, 20 mL of methanol/distilled water (10:90, v/v), 10 mL of methanol/distilled water (30:70, v/v), and 20 mL of methanol/distilled water (50:50, v/v) were used to remove undesired impurities. The product was eluted with additional with 40 mL of methanol (50:50, v/v). The product **13**/**14b** was isolated from the reaction mixture (160 mL) after an incubation period of 2 h (**1b**:**3e**, 1:1 mM assay). After charging the column with 40 mL of reaction mixture, 40 mL of methanol/distilled water (20:80, v/v) was used to remove undesired impurities. The product was eluted with 50 mL of methanol (50:50, v/v).

For nuclear magnetic resonance (NMR) spectroscopy, the isolated products were dried by lyophilization. The lyophilized products and reaction mixtures were characterized using an LC/MS system. The atmospheric pressure ionization (API) mass spectrometry experiments were performed on an Agilent Series 1200 HPLC system with a diode array detector and Agilent 6120 quadrupole mass spectrometer (Waldbronn, Germany). The high-resolution mass spectra (HRMS) were recorded on a QExactive classic system (Thermo Scientific, Bremen, Germany).

4a-Hydroxy-*N*-(2-hydroxyethyl)-2-oxo-10*H*-phenothiazine-1-carboxamide (5b). Synthesis and isolation as described above. Brown solid. Yield, 29.24% (29.77 mg). mp (decomposition) 160 °C. ^{1}H NMR (DMSO-d_6): δ 3.39 (m, J = 5.5, 2H, H-13), 3.53 (m, J = 5.2, J = 5.5, 2H, H-14), 4.77 (t, J = 5.2, 1H, OH, at C-14), 6.27 (d, J = 9.9, 1H, H-3), 6.82 (dd, J = 0.5, J = 9.9, 1H, H-4), 7.19 (m(t), J = 1.3, J = 7.6, 1H, H-7), 7.33 (m(t), J = 1.3, J = 8.0, 1H, H-8), 7.38 (m(d), J = 1.3, J = 8.0, 1H, H-9), 7.46 (m(d), J = 1.3, J = 7.6, 1H, H-6), 7.53 (s(broad), 1H, OH, at C-4a), 10.09 (t, J = 5.5, 1H, NH, at N-12), 14.56 (s, 1H, NH, at N-10). ^{13}C NMR: δ 41.6 (C-13), 60.1 (C-14), 69.6 (C-4a), 94.8 (C-1), 116.3 (C-5a), 119.7 (C-9), 125.6 (C-7), 127.9 (C-8), 128.8 (C-6), 129.8 (C-3), 134.6 (C-9a), 137.8 (C-4), 157.4 (C-10a), 169.4 (C-11), 182.4 (C-2). HMBC correlations: H-3 (C-1, C-4, C-4a, C-11), H-4 (C-2, C-4a, C-5a, C-10a), H-6 (C-8, C-9a), H-7 (C-5a, C-6, C-9), H-8 (C-5a, C-6, C-9a), H-9 (C-5a, C-7), H-10 (C-1, C-4a, C-5a, C-9, C-9a, C-10a), H-12 (C-1, C-11, C-13), H-13 (C-11, C-14), H-14 (C-13), OH at C-14 (C-13, C-14). R_f (HPLC) 10.89 min; UV−vis (MeOH) λ_{max} 252, 284, 300, 397 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 319.0 (100); AP-ESI: neg. ion mode $[M - H]^-$, 316.9 (100). HRMS (ESI/Quadrupole-Orbitrap) m/z: $[M + H]^+$ calcd for $C_{15}H_{15}N_2O_4S$, 319.0753; found, 319.0746.

4a-Hydroxy-*N*-(2-hydroxyethyl)-2-oxo-10*H*-phenothiazine-1-carboxylic Acid Methylester (5c). Synthesis and isolation as described above. Orange solid. Mixture of **8c**, **5c**, and other inseparable substances. Yield, 15.56% (18.01

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mg). The peak areas of the integrated ^{1}H NMR signals are in a 1:1:1 ratio, so the yield distribution between **8c**, **5c**, and the other inseparable substances was given as 5.19, 5.19, and 5.19%. ^{1}H NMR (DMSO-d_6), **5c**: δ 3.76 (s, 3H, H-12), 6.16 (d, J = 10.0, 1H, H-3), 6.77 (m(d), J = 10.0, J = 0.5, 1H, H-4), 7.11 (m(t), J = 7.5, J = 1.1, 1H, H-7), 7.27 (m(t), J = 7.9, J = 7.4, J = 1.3, 1H, H-8), 7.38 (m(d), J = 8.1, J = 1.1, 1H, H-6), 7.42 (m(d), J = 8.1, J = 1.0, 1H, H-9), 7.50 (s, 1H, OH, at C-4a), 11.42 (s, 1H, NH, H-10). ^{13}C NMR, **5c**: δ 57.3 (C-12), 69.7 (C-4a), 97.6 (C-1), 116.4 (C-5a), 119.3 (C-9), 125.0 (C-7), 127.7 (C-8), 128.8 (C-6), 129.9 (C-3), 135.2 (C-9a), 137.8 (C-4), 154.6 (C-10a), 168.9 (C-11), 179.6 (C-2). HMBC correlations, **5c**: H-3 (C-1, C-4a), H-4 (C-2, C-4a, C-10a), H-6 (C-8, C-9a), H-7 (C-5a, C-8, C-9), H-8 (C-5a, C-6, C-9a), H-9 (C-5a, C-7), H-12 (C-11), OH at C-4a (C-4a, C-10a), H-10 (C-1, C-4a, C-5a, C-9, C-9a). R_f (HPLC) 11.13 min; UV−vis (MeOH) λ_{max} 249, 280, 300, 382 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 290.1 (100); AP-ESI: neg. ion mode $[M - H]^-$, 288.0 (100).

[2-[(2-Aminophenyl)disulfanyl]anilino]-3,6-dihydroxy-benzoic Acid (8c). Mixture of **8c** and **5c**. ^{1}H NMR (DMSO-d_6), δ structure part of laccase substrate **1c** within **8c**: 3.60 (s, 3H, H-14), 6.62 (d, J = 8.8, 1H, H-5), 6.95 (s, 1H, NH, H-15), 6.89 (d, J = 8.8, 1H, H-4), 8.99 (s, 1H, OH at C-3), 9.34 (s, 1H, OH at C-6); structure part of the dimer **3** within **8c**: 6.39 (m(d), J = 8.2, J = 1.0, 1H), 6.42 (m(t), J = 7.5, J = 7.3, J = 1.1, 1H), 6.60 (m(t), J = 8.2, J = 1.1, 1H), 6.72 (m(d), J = 8.1, J = 1.1, 1H), 6.73 (m(d), J = 8.2, J = 1.4, 1H), 7.00 (m(d), J = 7.7, J = 1.5, 1H), 7.07 (m(t), J = 7.6, J = 1.3), 7.09 (m(t), 1H). ^{13}C NMR, δ structure part of laccase substrate **1c** within **8c**: 52.4 (C-14), 113.5 (C-5), 117.1 (C-1), 119.7 (C-4), 126.8 (C-2), 144.8 (C-3), 149.3 (C-6), 167.7 (C-13); structure part of the dimer **6** within **8c**: 114.9 (1H 6.39), 115.3x (1H 6.72, 1H 6.73), 116.x (1H 6.42), 118.8 (1H 6.60), 131.6 (1H 7.07), 134.6 (1H 7.09), 135.9 (1H 7.00). HMBC correlations, structure part of laccase substrate **1c** within **8c**: H-4 (C-1, C-2, C-3, C-6), H-5 (C-1, C-3, C-6, C-13), H-14 (C-13), OH at C-3 (C-2, C-3, C-4), OH at C-6 (C-1, C-5, C-6), H-15 (C-3, C-8, C12); structure part of the dimer **6** within **8c**: 6.42 (115.3x, 131.6, 135.9), 6.60 (120.2, 134.6), 6.72 (116.x), 6.73 (116.x), 7.00 (131.6, 150.2), 7.07 (115.3x, 117.1, 135.9, 150.2), 7.09 (131.6).

3-Hydroxy-*N*-(2-hydroxyethyl)-10*H*-phenothiazine-1-carboxamide (9b) and *N*-(2-Hydroxyethyl)-3-oxo-phenothiazine-1-carboxamide (10b). Synthesis and isolation as described above. Violet solid. Yield, 6.39% (6.18 mg). mp 209 °C. ^{1}H NMR (DMSO-d_6), **9b**: δ 3.30 (signal covered by the solvent signal, H-13), 3.54 (t, J = 6.0, 2H, H-14), 6.64 (d(s), J = 2.5, 1H, H-4), 6.66 (m(d), J = 8.0, J = 1.1, 1H, H-9), 6.80 (m(t), J = 7.5, J = 1.1, 1H, H-7), 6.97 (m(d), J = 7.6, J = 1.2, 1H, H-6), 7.00 (d(s), J = 2.6, 1H, H-2), 7.01 (m(t), J = 7.6, J = 1.2, 1H, H-8), 8.49 (t, J = 5.2, 1H, NH, H-12), 9.22 (s(broad), 1H, OH), 9.81 (s, 1H, NH, H-10). ^{13}C NMR, **9b**: δ 42.6 (C-13), 60.0 (C-14), 113.3 (C-2), 115.6 (C-9), 116.9 (C-5a), 117.1 (C-4), 120.1 (C-1), 122.5 (C-7), 126.7 (C-6), 128.1 (C-8), 135.6 (C-10a), 142.2 (C-9a), 152.4 (C-3), 168.5 (C-11). HMBC correlations, **9b**: H-2 (C-3, C-4, C-10a, C-11), H-4 (C-2, C-3, C-10a), H-6 (C-8, C-9a), H-7 (C-5a, C-6, C-9), H-8 (C-6, C-9a), H-9 (C-5a, C-7), H-10 (C-1, C-5a, C-9), H-12 (C-11), H-13 (C-11, C-14), H-14 (C-13). **10b**: R_f (HPLC) 8.86 min; R_f (LC/MS) 12.95 min; UV−vis (MeOH) λ_{max} 234, 293, 385, 539 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 301.0 (100); $[2M + Na]^+$, 622.9 (5). **9b**: MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 303.0 (5).

4a-Hydroxy-*N*-(2-hydroxyethyl)-2-oxo-10*H*-phenoxazine-1-carboxamide (11b). Synthesis and isolation as described above. Orange solid. Yield, 8.40% (7.06 mg). mp (decomposition) 165 °C. ^{1}H NMR (DMSO-d_6): δ 3.38 (m, J = 5.6 Hz, 2H, H-13), 3.53 (m, J = 5.8, 2H, H-14), 4.77 (t, J = 4.8, 1H, OH, at C-14), 6.22 (d, J = 10.1, 1H, H-3), 6.92 (d, J = 10.0, 1H, H-4), 7.11 (m, J = 7.6, J = 7.8, 1H, H-8), 7.14 (m, J = 7.5, 1H, H-6), 7.16 (m, J = 7.5, J = 7.6, 1H, H-7), 7.43 (d, J = 7.8, 1H, H-9), 8.20 (s, 1H, OH, at C-4a), 9.74 (t, J = 5.5, 1H, NH, H-12), 13.71 (s, 1H, NH, H-10). ^{13}C NMR: δ 41.4 (C-13), 60.2 (C-14), 86.8 (C-4a), 95.8 (C-1), 118.1 (C-6), 118.3 (C-9), 123.5 (C-8), 125.8 (C-9a), 125.9 (C-7), 129.8 (C-3), 137.5 (C-4), 140.6 (C-5a), 156.1 (C-10a), 168.8 (C-11), 182.8 (C-2). HMBC correlations: H-3 (C-1, C-4a, C-11), H-4 (C-1, C-2, C-10a), H-6 (C-9a), H-7 (C-5a, C-9), H-8 (C-6, C-9a), H-9 (C-5a, C-7), H-10 (C-1, C-4a, C-5a, C-9, C-9a, C-10a), H-12 (C-1, C-11, C-13, C-14), H-13 (C-11, C-14), H-14 (C-13), OH at C-14 (C-13, C-14), OH at C-4a (C-4, C-4a, C-10a). ^{1}H-^{1}H COSY correlations: H-3 (H-4), H-4 (H-3), H-6 (H-7), H-7 (H-6), H-8 (H-9), H-9 (H-8), H-10 (H-4, H-6, H-9), H-12 (H-13, OH at C-14), H-13 (H-12, H-14), H-14 (H-13, OH at C-14), OH at C-14 (H-14), OH at C-4a (H-3, H-4, H-9). R_f (HPLC) 10.20 min; UV−vis (MeOH) λ_{max} 208, 283, 387 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 303.0 (100); $[M + Na]^+$, 324.9 (9); $[2M + H]^+$, 626.9 (7). HRMS (ESI/Quadrupole-Orbitrap) m/z: $[M + H]^+$ calcd for $C_{15}H_{15}N_2O_5$, 303.0981; found 303.0972; $[M + Na]^+$ calcd for $C_{15}H_{14}N_2O_5Na$, 325.0800; found, 325.0792.

2-Hydroxy-*N*-(2-hydroxyethyl)-phenazine-1-carboxamide (12b). Synthesis and isolation as described above. Yellow solid. Yield, 71.01% (48.28 mg). mp (decomposition) 170 °C. ^{1}H NMR (DMSO-d_6): δ 3.63 (m, J = 5.3, 2H, H-13), 3.76 (m, J = 5.3, 2H, H-14), 5.09 (s(broad), 1H, OH, at C-14), 7.56 (d, J = 8.9, 1H, H-3), 7.89 (m(t), J = 7.5, 1H, H-8), 7.98 (m(t), J = 1.0 Hz, J = 7.5, 1H, H-7), 8.18 (d, J = 8.7, 3H, H-4, H-6, H-9), 11.78 (s, 1H, NH, at N-12). ^{13}C NMR (DMSO-d_6): δ 42.0 (C-13), 59.9 (C-14), 102.6 (C-1), 128.4 (C-3, C-6), 129.6 (C-9), 130.1 (C-8), 132.4 (C-7), 135.7 (C-4), 139.9 (C-4a), 140.5 (C-5a, C-9a), 142.1 (C-2), 169.6 (C-10a), 170.8 (C-11). HMBC correlations: H-3 (C-1, C-4a), H-4 (C-2, C-10a), H-6 (C-8, C-9a), H-7 (C-5a, C-9), H-8 (C-6, C-9a), H-9 (C-5a, C-7). R_f (HPLC) 12.66 min; UV−vis (MeOH) λ_{max} 213, 252, 360, 403 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 284.0 (100). HRMS (ESI/Quadrupole-Orbitrap) m/z: $[M + H]^+$ calcd for $C_{15}H_{14}N_3O_3$, 284.1035; found, 284.1031.

2-Hydroxyphenazine-1-carboxylic Acid Methylester (12c). Synthesis and isolation as described above. Red solid. Yield, 6.60% (10.31 mg). ^{1}H NMR (DMSO-d_6): δ 3.96 (s, 3H, H-12), 7.68 (d, J = 9.4, 1H, H-3), 7.85 (m, J = 8.5, J = 6.6, J = 1.3, 1H, H-8), 7.91 (m, J = 8.5, J = 6.6, J = 1.3, 1H, H-7), 8.10 (d, J = 8.5, J = 0.7, 1H, H-6), 8.18 (d, J = 9.4, 1H, H-4), 8.19 (d, J = 8.5, J = 0.7, 1H, H-9). ^{13}C NMR (DMSO-d_6): δ 52.5 (C-12), 113.9 (C-1), 126.9 (C-3), 129.0 (C-6), 129.7 (C-8), 129.9 (C-9), 131.6 (C-7), 132.3 (C-4), 138.9 (C-4a), 141.3 (C-9a), 142.5 (C-2), 143.0 (C-5a), 158.0 (C-10a), 167.4 (C-11). HMBC correlations: H-3 (C-1, C-4a), H-4 (C-2, C-10a), H-6 (C-8, C-9a), H-7 (C-5a, C-9), H-8 (C-6, C-9a), H-9 (C-5a, C-7). R_f (HPLC) 11.88 min; UV−vis (MeOH) λ_{max} 217, 247, 360, 470 nm. MS m/z (rel. intensity) AP-ESI: pos. ion

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mode $[M + H]^+$, 255.1 (100); AP-ESI: neg. ion mode $[M - H]^-$, 253.0 (100).

***N*-(2-Hydroxyethyl)-2-(2-methylanilino)-3,6-dioxocyclohexa-1,4-diene-1-carboxamide (13a) and 3,6-Dihydroxy-*N*-(2-hydroxyethyl)-2-(2-methylanilino)-benzamide (14a).** Synthesis and isolation as described above. Orange solid. Yield, 29.14% (10.50 mg). mp 156 °C. The peak area of the integrated ^{1}H NMR signals are in a 1:1 ratio, so the yield distribution between **13a** and **14a** is given as 14.57 and 14.57%. **13a**: *N*-(2-Hydroxyethyl)-2-(2-methylanilino)-3,6-dioxo-cyclohexa-1,4-diene-1-carboxamide; ^{1}H NMR (DMSO-d_6): δ 2.21 (s, 3H, H-8′), 3.23 (m, J = 5.6, 2H, H-9), 3.46 (m, J = 5.5, 2H, H-10), 4.76 (m, J = 5.1, 1H, OH at C-10), 6.72 (d, J = 10.1, 1H, H-4), 6.74 (d, J = 10.1, 1H, H-5), 7.12−7.17 (3 x m, 3H, H-3′, H-4′, H-5′), 7.25 (d, J = 7.4, 1H, H-6′), 9.40 (t(broad), 1H, H-8, NH). ^{13}C NMR (DMSO-d_6): δ 17.5 (C-8′), 41.0 (C-9), 59.3 (C-10), 102.4 (C-1), 125.3/126.1, (C-3′/C5′), 126.3 (C-4′), 130.2 (C-6′), 132.0 (C-1′), 133.2 (C-4), 138.1 (C-2′), 139.6 (C-5), 151.0 (C-2), 167.5 (C-7), 182.8 (C-3), 183.6 (C-6). HMBC correlations: H-4 (C-2, C-6), H-5 (C-1, C-3, C-7), H-8 (C-7, C-9), H-9 (C-7, C-10), H-10 (C-9), H-3′/H-4′/H-5′ (C-2′, C-3′, C-5′), H-6′ (C-2′, C-4′, C-8′), H-8′ (C-1′, C-2′, C-6′), OH at C-10 (C-9, C-10). R_f (HPLC) 8.89 min; UV−vis (MeOH) λ_{max} 211, 260, 490 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 301.0 (100). HRMS (ESI/Quadrupole-Orbitrap) m/z: $[M + H]^+$ calcd for $C_{16}H_{17}N_2O_4$, 301.1188; found, 301.1182; $[M + Na]^+$ calcd for $C_{16}H_{16}N_2O_4Na$, 323.1008; found, 323.1001. **14a**: 3,6-Dihydroxy-*N*-(2-hydroxyethyl)-2-(2-methylanilino)-benzamide: ^{1}H NMR (DMSO-d_6): δ 2.29 (s, 3H, H-8′), 3.18 (m, J = 6.1, J = 5.5, 2H, H-9), 3.24 (m, J = 5.5, 2H, H-10), 4.64 (m, J = 5.2, 1H, OH at C-10), 6.23 (d, J = 7.9, 1H, H-3′), 6.62 (d, J = 8.9, 1H, H-5), 6.68 (m, J = 7.3, J = 0.6, 1H, H-5′), 6.91 (m, J = 7.5, J = 0.6, 1H, H-4′), 6.93 (d, J = 8.9, 1H, H-4), 7.03 (s, 1H, H-7′, NH), 7.07 (d, J = 7.2, 1H, H-6′), 8.88 (s, 1H, OH), 8.98 (t, J = 5.4, 1H, H-8, NH), 11.25 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ 17.4 (C-8′), 41.2 (C-9), 59.1 (C-10), 112.9 (C-5), 113.3 (C-3′), 113.7 (C-1), 119.0 (C-5′), 119.7 (C-4), 124.6 (C-1′), 126.0 (C-4′), 128.4 (C-6), 129.7 (C-6′), 143.7 (C-2′), 144.2 (C-3), 151.6 (C-2), 168.0 (C-7). HMBC correlations: H-4 (C-1, C-2, C-3, C-6), H-5 (C-1, C-2, C-3, C-6, C-7), H-8 (C-7, C-9, C-10), H-9 (C-7, C-10), H-10 (C-9), H-3′ (C-1′, C-2′, C-5′), H-4′ (C-2′, C-6′), H-5′ (C-1′, C-3′), H-6′ (C-2′, C-4′, C-8′), H-7′ (C-3, C-3′), H-8′ (C-1′, C-2′, C-3′, C-6′), OH at C-10 (C-9, C-10), OH (C-3, C-4, C-6), OH (C-1, C-2).

***N*-(2-Hydroxyethyl)-2-(2-nitroanilino)-3,6-dioxocyclohexa-1,4-diene-1-carboxamide (13b) and 3,6-Dihydroxy-*N*-(2-hydroxyethyl)-2-(2-nitroanilino)benzamide (14b).** Synthesis and isolation as described above. Brown solid. Yield, 15.53% (8.23 mg). **14b**: 3,6-Dihydroxy-*N*-(2-hydroxyethyl)-2-(2-nitroanilino)benzamide: ^{1}H NMR (DMSO-d_6): δ 3.16 (m, J = 5.9, 2H, H-9), 3.44 (m, 2H, H-10), 4.49 (s(broad), 1H, OH at C-10), 6.55 (m(d), J = 1.1, J = 8.7, 1H, H-3′), 6.72 (d, J = 8.3, 1H, H-5), 6.77 (m, J = 7.7, J = 1.2, 1H, H-5′), 6.85 (d, J = 8.3, 1H, H-4), 7.39 (m, J = 7.8, J = 1.4, 1H, H-4′), 8.05 (m, J = 1.4, J = 8.6, 1H, H-6′), 8.15 (t, J = 5.5, 1H, H-8, NH), 9.03 (s(broad), 1H, OH), 9.38 (s, 1H, H-7′, NH), 9.65 (s(broad), 1H, OH). ^{13}C NMR (DMSO-d_6): δ 41.9 (C-9), 59.9 (C-10), 115.5 (C-5), 117.7 (C-3′), 121.7 (C-1), 117.5 (C-5′), 118.6 (C-4), 123.7 (C-6), 133.2 (C-1′), 135.9 (C-4′), 125.8 (C-6′), 143.4 (C-2′), 145.5 (C-3), 148.7 (C-2), 166.3 (C-7). HMBC correlations: H-4 (C-2, C-3, C-5, C-6), H-5 (C-1, C-2, C-3, C-7), H-8 (C-7), H-9 (C-7, C-10), H-10 (C-9), H-3′ (C-1′, C-5′), H-4′ (C-2′, C-6′), H-5′ (C-1′, C-3′), H-6′ (C-1′, C-2′, C-4′), H-7′ (C-1, C-1′, C-3, C-3′). R_f (HPLC) 9.00 min; UV−vis (MeOH) λ_{max} 209, 262, 483 nm. MS m/z (rel. intensity) AP-ESI: pos. ion mode $[M + H]^+$, 331.9 (100); $[M + Na]^+$, 353.9 (7).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00719.

^{1}H NMR, ^{13}C NMR, HSQC, HMBC spectra, ^{1}H-NMR and ^{13}C-NMR correlation, retention time, UV−vis, and MS-data (PDF)

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Notes

The authors declare no competing financial interest.

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Not applicable.

■ DEDICATION

[‖]This paper is dedicated to the memory of Prof. Dr. Frieder Schauer.

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